# *Pseudomonas Aeruginosa*- and IL-1β-Mediated Induction of Human β-Defensin-2 in Keratinocytes Is Controlled by NF-κB and AP-1

Kai Wehkamp[1], Lars Schwichtenberg[1], Jens-Michael M. Schröder[1] and Jürgen Harder[1]

Human β-defensin-2 (hBD-2) is an inducible epithelial peptide antibiotic involved in cutaneous defense. Expression of hBD-2 in keratinocytes is strongly induced by IL-1β and culture supernatants of *Pseudomonas aeruginosa* (PA). The use of an IL-1 receptor antagonist revealed that PA-mediated induction of hBD-2 is not dependent on IL-1. Luciferase gene reporter experiments, demonstrated that a 2,338 bp promoter fragment of hBD-2 containing three putative NF-κB as well as one activator protein-1 (AP-1) binding site was strongly activated by IL-1β and PA. Mutation of all NF-κB binding sites together with mutation of the AP-1 binding site completely abolished hBD-2 promoter activation by IL-1β and PA. Treatment with the NF-κB inhibitor Helenalin as well as with the c-Jun N-terminal kinase (JNK) inhibitor SP600125 and the p38 mitogen-activated protein kinase inhibitor SB 202190 blocked hBD-2 induction by IL-1β and PA. PD 98059, a selective inhibitor of extracellular signal-regulated kinase 1/2 demonstrated no significant influence. Transcription factor ELISAs indicated that the NF-κB heterodimer p50–p65 binds to all three NF-κB sites in the hBD-2 promoter upon stimulation of primary keratinocytes with IL-1β and PA. We conclude that the activation of NF-κB (p50–p65) and AP-1 are crucial events for induction of hBD-2 in keratinocytes upon IL-1β and PA stimulation.

*Journal of Investigative Dermatology* (2006) **126,** 121–127. doi:10.1038/sj.jid.5700020

## INTRODUCTION

Human skin is in permanent contact with various potential pathogenic microorganisms. To overcome these microbial threats, the skin has developed a chemical defense system that is based on the production of various antimicrobial proteins. The *in vivo* relevance of antimicrobial proteins in cutaneous host defense has been demonstrated in a mouse model. Mice, deficient in the expression of the cathelicidin CRAMP (the mouse homolog of the human cathelicidin LL-37), were more susceptible to skin infections caused by group A *Streptococcus*. Group A *Streptococcus* mutants resistant to CRAMP resulted in more severe skin infections in normal mice (Nizet *et al.*, 2001). Recently, *in vivo* experiments with neutralizing anti-psoriasin antibodies identified psoriasin (S100A7) as a key factor in the resistance of human skin against *E. coli* (Glaeser *et al.*, 2005).

Psoriatic skin represents a particularly rich source of antimicrobial proteins (Harder and Schroeder, 2005), thus offering an explanation as to why patients with psoriasis suffer fewer cutaneous bacterial infections than expected (Christophers and Henseler, 1987). In contrast, the expression of the antimicrobial proteins human β-defensin-2 (hBD-2), hBD-3, and LL-37 is decreased in acute and chronic lesions from patients suffering from atopic dermatitis (Ong *et al.*, 2002; Nomura *et al.*, 2003). It is believed that the low expression of antimicrobial proteins may at least partially account for the increased susceptibility of patients with atopic dermatitis to skin infection.

hBD-2 was the first inducible human antimicrobial protein and was originally isolated from lesional psoriatic skin (Harder *et al.*, 1997). hBD-2 belongs to the β-defensin family, a group of small (4–5 kDa), cationic peptide antibiotics that were first discovered in cattle (Diamond *et al.*, 1991). hBD-2 exhibits a broad spectrum of antimicrobial activity and its capacity to kill bacteria *in vivo* has been demonstrated in a mouse gene therapy study with hBD-2-transfected tumor cells. Following a bacterial infection, mice with hBD-2-bearing tumors bore fewer viable bacteria than controls (Huang *et al.*, 2002). Expression of hBD-2 in epithelial cells is induced by proinflammatory cytokines and bacteria (Schroeder and Harder, 1999). The inducibility of hBD-2 may explain why hBD-2 expression is increased in inflammed skin (Liu *et al.*, 1998). Endogenous proinflammatory cytokines like IL-1 and exogenous stimuli such as *Pseudomonas aeruginosa* (PA) have proven to be the most effective inducers of hBD-2 in keratinocytes

[1]*Clinical Research Unit, Department of Dermatology, University Hospital Schleswig-Holstein, Campus Kiel, Germany*

*Correspondence: Dr Jürgen Harder, Clinical Research Unit, Department of Dermatology, University Hospital Kiel, Schittenhelmstr. 7, Kiel D-24105, Germany. E-mail: jharder@dermatology.uni-kiel.de*

*Abbreviations: AP-1, activator protein-1; hBD-2, human β-defensin-2; IL-1ra, IL-1 receptor antagonist; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; PA,* Pseudomonas aeruginosa*; PBS, phosphate-buffered saline*

*Received 25 May 2005; revised 23 August 2005; accepted 29 August 2005*

(Harder *et al.*, 1997, 2004; Liu *et al.*, 2002; Sorensen *et al.*, 2003, 2005).

Little has been described to date regarding the gene regulation of hBD-2 induction by either IL-1 or PA in keratinocytes. Therefore, our goal was to characterize the signaling pathways involved in hBD-2 induction in response to its strong inducers PA and IL-1β.

## RESULTS

### Induction of hBD-2 in primary keratinocytes by PA and IL-1β

Primary keratinocytes were treated with either culture supernatants of PA or IL-1β, and hBD-2 expression was measured by real-time PCR and ELISA. As expected, PA as well as IL-1β induced hBD-2 gene and protein expression in primary keratinocytes (Figure 1a and b). To test whether hBD-2 induction correlates with hBD-2 promoter activation, 2,338 bp of the hBD-2 promoter was ligated in front of the *firefly* luciferase gene and this construct was used to transiently transfect primary keratinocytes. Stimulation of the transfected keratinocytes with PA or IL-1β led to a strong increase in luciferase activity, indicating activation of the hBD-2 promoter (Figure 2b and c).

### Induction of hBD-2 expression by PA is not mediated by autocrine IL-1 induction

Our analyses revealed no significant differences between IL-1β- and PA-mediated hBD-2 induction. Therefore, we wondered whether PA induces hBD-2 by an autocrine IL-1β- or IL-1α-mediated mechanism. We used an IL-1 receptor

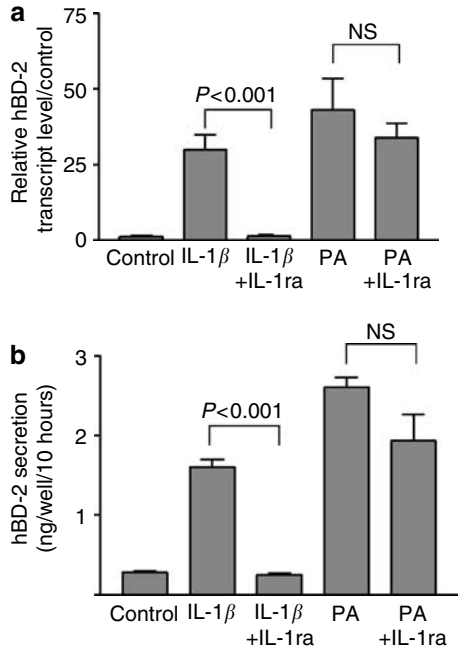


**Figure 1. HBD-2 induction by PA is not mediated via IL-1.** Primary keratinocytes were incubated with or without a specific IL-1-receptor antagonist (IL-1ra, 300 ng/ml) for 1 hour and subsequently stimulated with IL-1β or PA for 10 hours. (**a**) hBD-2 mRNA expression was analyzed by real-time RT-PCR and normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The relative transcript level was further normalized with the unstimulated control, which was assigned the value 1. (**b**) hBD-2 expression was measured in cell culture supernatants using an hBD-2-ELISA. Data are means ± SD of one representative experiment of three, each carried out in triplicate samples. Differences between IL-1ra-treated and -untreated cells were analyzed by two-tailed, nonpaired *t*-test, with $P<0.05$ indicative of statistical significance (NS = not significant).

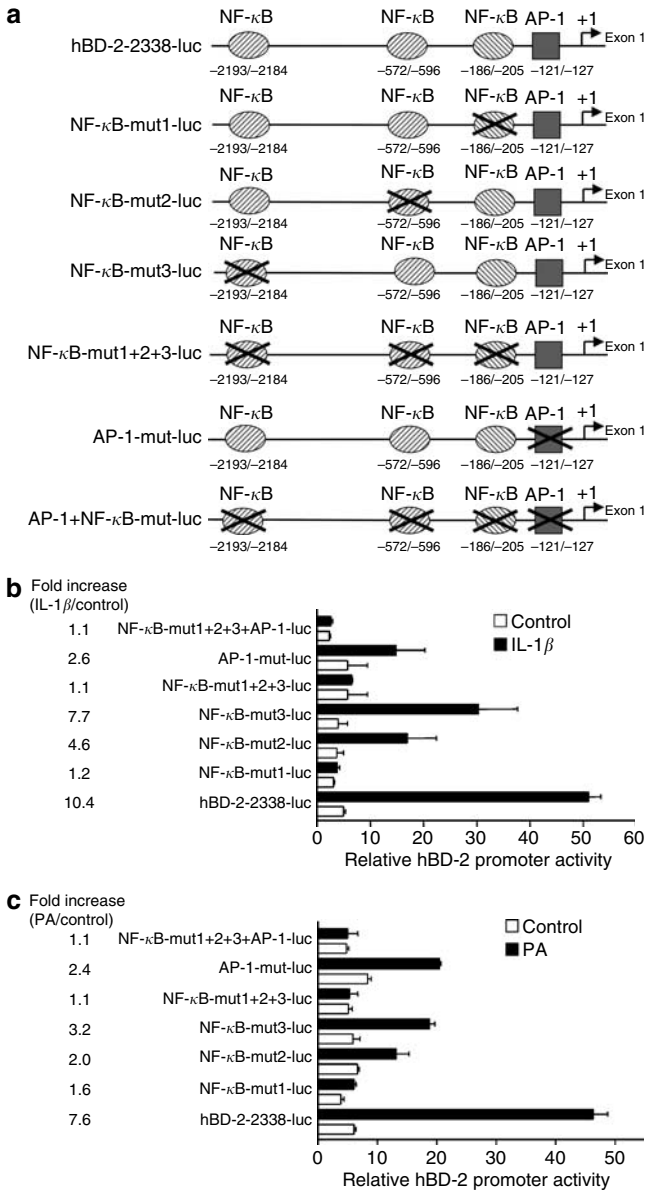


**Figure 2. Mutation of NF-κB and AP-1 binding sites inhibits IL-1β- and PA-mediated hBD-2 induction.** (**a**) The hBD-2 promoter constructs. Nucleotide positions are marked relative to the hBD-2 transcription start. Three NF-κB sites and one AP-1 site in the hBD-2 promoter (−2,338 to −1 bp) linked to the luciferase gene were mutated in different combinations. (**b** and **c**) Primary keratinocytes were transfected with the wild-type (−2,338-luc) or mutated hBD-2-promoter-luciferase plasmids together with an internal control (*renilla* luciferase expression plasmid). After transfection, cells were stimulated with IL-1β (**b**) or supernatants of PA (**c**) for 10 hours and relative hBD-2 promoter activity was determined as a ratio between *firefly* and *renilla* luciferase activity. Data are means ± SD of one representative experiment of three, each carried out in triplicate samples.

antagonist (IL-1ra) to determine the influence of IL-1 in keratinoctyes stimulated with PA. Primary keratinocytes preincubated with IL-1ra and subsequently stimulated with PA showed no significant reduction in hBD-2 induction as compared to non-IL-1ra-treated cells (Figure 1a and b). Abolishment of IL-1β-mediated hBD-2 expression by IL-1ra served as a positive control (Figure 1a and b).

**NF-κB and AP-1 binding sites in the hBD-2 promoter are required for PA- and IL-1β-mediated hBD-2 induction**

To analyze the functional importance of putative binding sites for the transcription factors NF-κB and activator protein-1 (AP-1) on PA- and IL-1β-mediated induction of hBD-2, we generated different hBD-2-promoter-luciferase constructs containing mutations of the three NF-κB binding sites (positions −205 to −186, −596 to −572, and −2,193 to −2,184) and the AP-1 binding site (positions −127 to −121) (Figure 2a). Interestingly, the influence of these four putative binding sites on hBD-2 induction in primary keratinocytes turned out to be similar for PA as well as IL-1β stimulation (Figure 2b and c). Mutation of all three NF-κB sites as well as mutation of the three NF-κB sites together with the AP-1 site almost completely abolished PA- and IL-1β-mediated hBD-2 promoter activation. Separate mutations of the three NF-κB binding sites revealed that the first proximal NF-κB binding site (positions −205 to −186) is of major importance for the IL-1β- and PA-mediated hBD-2 induction followed by the second (positions −596 to −572) and the third (positions −2,193 to −2,184) NF-κB binding site (Figure 2b and c). The potential AP-1 binding site (positions −127 to −121) is also crucial for full IL-1β- and PA-mediated hBD-2 induction because its mutation efficiently reduced hBD-2 promoter activation (Figure 2b and c).

**Inhibitors of MAP kinases and NF-κB pathways block PA- and IL-1β-mediated hBD-2 induction**

To verify the influence of the transcription factors NF-κB and AP-1 on PA- and IL-1β-mediated hBD-2 induction, we used inhibitors of the three AP-1 activating mitogen-activated protein kinase (MAP) kinase cascades (extracellular signal-regulated kinase 1/2 pathway, c-Jun N-terminal kinase (JNK) pathway, and p38 pathway) as well as the NF-κB inhibitor Helenalin. Treatment with SB 202190, a specific p38 MAP kinase inhibitor, as well as treatment with SP600125, a selective inhibitor of JNK, strongly reduced hBD-2 protein induction by PA and IL-1β in primary keratinocytes (Figure 3). PD 98059, a selective inhibitor of extracellular signal-regulated kinase 1/2 did not decrease IL-1β-mediated hBD-2 induction but moderately reduced PA-mediated hBD-2 induction (Figure 3). Furthermore, hBD-2 gene induction by PA or IL-1β was also blocked by treatment of primary keratinocytes with the NF-κB inhibitor Helenalin (Figure 3). To measure potential cytotoxic effects of the inhibitors on the keratinocytes, we determined the release of lactate dehydrogenase using the Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany). The MAP kinase inhibitors showed no significant lactate dehydrogenase release and Helenalin showed only a very low increase in lactate dehydrogenase release (5%), indicating no major cytotoxic effects. In addition, expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was not affected by the inhibitors, whereas changes in hBD-2 gene expression correlated with hBD-2 protein release (data not shown).

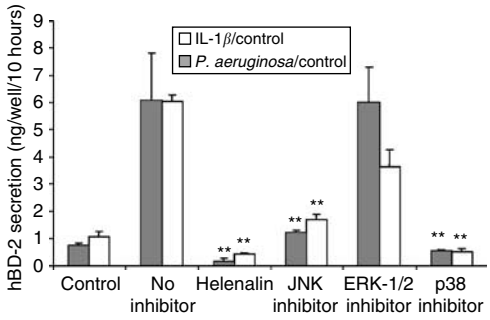


**Figure 3. Inhibition of *P. aeruginosa*- or IL-1β-mediated hBD-2 induction in primary keratinocytes by NF-κB, JNK, and p38-MAP kinase inhibitor.** Primary keratinocytes were preincubated for 1 hour with inhibitors for NF-κB and the MAP kinases, extracellular signal-regulated kinase (ERK)1/2, JNK, and p38. Subsequent stimulation was performed using supernatants of PA or IL-1β for 10 hours. hBD-2 expression was measured in cell culture supernatants using an hBD-2-ELISA. Data are means ± SD of one representative experiment of three, each carried out in triplicate samples. **Significantly different from stimulated cells without inhibitor treatment ($P < 0.01$, Student's *t*-test).

**Identification of NF-κB family members p65 and p50 bound to the three NF-κB binding sites in the hBD-2 promoter upon stimulation with PA or IL-1β**

Since the NF-κB transcription factor consists of homo- or heterodimeric subunits of the NF-κB/Rel family, we determined the NF-κB subunits bound to the hBD-2 promoter in IL-1β and PA-stimulated primary keratinocytes. Using a transcription factor ELISA, we could show that binding of the subunits p65 and p50 to all three NF-κB binding sites of the hBD-2 promoter was strongly increased in IL-1β- and PA-stimulated keratinoctyes (Figure 4). Binding of the NF-κB subunits p52, RelB, and C-Rel was not markedly increased (Figure 4).

## DISCUSSION

Recent studies have indicated that the production of skin-derived antimicrobial proteins provides a powerful defense mechanism to protect the epidermis against harmful microorganisms. Interestingly, the production of antimicrobial proteins in keratinocytes can be activated through endogenous signals such as proinflammatory cytokines and growth factors as well as through contact with bacteria (Ong *et al.*, 2002; Nomura *et al.*, 2003; Sorensen *et al.*, 2003, 2005; Harder *et al.*, 2004). hBD-2 represents one member of those inducible endogenous antimicrobial proteins. Expression of hBD-2 in keratinocytes is strongly induced by the proinflammatory cytokine IL-1β and bacteria such as PA (Sorensen *et al.*, 2003; Harder *et al.*, 2004). In agreement with these findings, we show here that IL-1β as well as supernatant of cultured PA activated a hBD-2-promoter-luciferase construct transfected in primary keratinocytes. Recent studies suggested that the epidermal response to microbe-derived molecules is

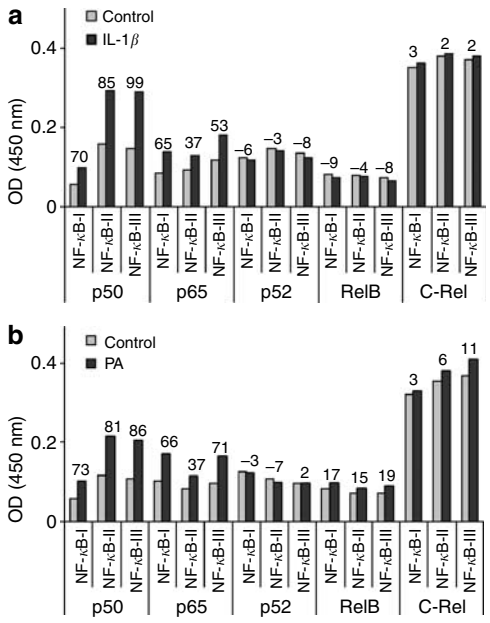


**Figure 4. Differential binding of p50, p52, p65, RelB, and C-Rel to three NF-κB binding sites in hBD-2 promoter.** Nuclear extracts of IL-1β- (**a**) or PA- (**b**) treated primary keratinocytes were incubated with different oligonucleotides each containing one of the putative NF-κB sites of the hBD-2 promoter (NF-κB-I at positions −205 to −186, NF-κB-II at positions −596 to −572, and NF-κB-III at positions −2,193 to −2,184). Binding of NF-κB subunits was analyzed using a transcription factor ELISA with specific antibodies for RelA, RelB, C-Rel, p50, and p52. Bars indicate the optical density (OD) at 450 nm and values indicate the relative (%) OD increment of IL-1β- (**a**) or PA- (**b**) treated cells compared to untreated control cells. Similar results were obtained with primary keratinocytes from two other donors.

potently amplified by monocytes and lymphocytes through IL-1-mediated signaling, leading to an increase in the production of hBD-2 by keratinocytes (Liu *et al.*, 2003). As it is known that keratinocytes are a rich source of IL-1 (Kupper and Groves, 1995; Grone, 2002), one may speculate that the production of hBD-2 by keratinocytes upon contact with PA is mediated by the release of IL-1 in an autocrine or paracrine manner. To investigate this possible IL-1-dependent induction of hBD-2 by PA, we used an IL-1 receptor antagonist. Treatment of the keratinocytes with the IL-1 receptor antagonist completely abolished IL-1β-mediated hBD-2 induction, whereas PA-mediated hBD-2 induction was not significantly affected. These data suggest that PA does not induce hBD-2 expression in keratinocytes through the autocrine or paracrine production of IL-1.

It is known that both IL-1β and bacteria can activate the transcription factors NF-κB and AP-1 (Hobbie *et al.*, 1997; Florin *et al.*, 2004; Vitiello *et al.*, 2004; Kelly and Conway, 2005). Since the promoter region of the hBD-2 gene contains binding sites for NF-κB as well as for AP-1, we analyzed the relevance of these binding sites for both IL-1β- and PA-mediated hBD-2 induction. Indeed, mutation of all three NF-κB sites almost completely blocked hBD-2 promoter activation upon treatment with either IL-1β or PA, indicating that NF-κB play a crucial role in the regulation of IL-1β- and PA-mediated hBD-2 gene induction. The importance of NF-κB in the signal transduction pathway leading to hBD-2 gene induction by IL-1β and PA was further confirmed by the observation that the specific NF-κB inhibitor Helenalin abolished hBD-2 induction upon treatment of the keratinocytes with either IL-1β or PA. The observation that hBD-2 induction in keratinocytes by PA or IL-1β is dependent on NF-κB is consistent with previous reports showing that NF-κB is a transcription factor essential for induction of hBD-2 by various bacteria. For example, induction of hBD-2 in colon or gastric epithelial cells by *Salmonella*, *E. coli*, and *Helicobacter pylori* is mediated by NF-κB (O'Neil *et al.*, 1999; Ogushi *et al.*, 2001; Wada *et al.*, 2001; Wehkamp *et al.*, 2004).

Separate mutations of the three NF-κB binding sites in the hBD-2 promoter revealed that all three NF-κB binding sites are essential for full induction of the hBD-2 gene upon IL-1β and PA stimulation. In particular, the first proximal NF-κB binding site (positions −205 to −186) plays an important role for IL-1β- and PA-mediated hBD-2 induction in keratinocytes because mutation of this site strongly inhibited hBD-2 promoter activation upon IL-1β and PA treatment. We observed the binding of the NF-κB subunits p65 and p50 to all three NF-κB binding sites, indicating that the IL-1β- and PA-mediated induction of hBD-2 involved the binding of the heterodimer p65–p50 to all three NF-κB binding sites of the hBD-2 promoter. The binding of the NF-κB heterodimer p65–p50 to the first proximal NF-κB binding site in the hBD-2 promoter has also been observed in mononuclear phagocytes stimulated with LPS and in A549 lung epithelial cells stimulated with IL-1β or tumor necrosis factor-α (Tsutsumi-Ishii and Nagaoka, 2002, 2003). In contrast, *Salmonella enteritidis* flagella filament protein induced hBD-2 expression in Caco-2 cells via p65–p65 homodimer binding to the first proximal NF-κB binding site (Ogushi *et al.*, 2001). In gastric MKN45 cells, only the p65–p65 homodimer was found to bind to the NF-κB site of the hBD-2 promoter upon *H. pylori* stimulation (Wada *et al.*, 2001). Therefore, it seems that the first proximal NF-κB binding site is able to bind both the p65–p65 homodimer as well as the p65–p50 heterodimer. All these reports together indicate that the first proximal NF-κB binding site represents the crucial region of the hBD-2 promoter with regard to NF-κB-mediated hBD-2 induction.

Recent studies have reported on hBD-2 induction by bacteria without the involvement of the transcription factor NF-κB. For example, Dale and co-workers demonstrated that hBD-2 induction with a periodontal bacterium *Fusobacterium nucleatum* in human gingival epithelial cells was not blocked by NF-κB inhibitors. They found that hBD-2 induction by *F. nucleatum* is regulated via the p38 and JNK kinase pathways and speculated that the activation of the p38 and JNK may regulate gene expression of hBD-2 through transcription factor AP-1 (Krisanaprakornkit *et al.*, 2002). As shown here, the proximal AP-1 binding site in the hBD-2 promoter is crucial for full induction of hBD-2 in keratinocytes by IL-1β and PA. It is known that AP-1 can be activated by different MAP kinase pathways including the ERK, the JNK pathway, and the p38 MAP kinase (Whitmarsh and Davis, 1996). Using specific inhibitors for these three MAP kinase pathways, we found that both the p38 and JNK kinase

pathways are involved in the induction of hBD-2 by both IL-1β and PA in keratinocytes.

It is an interesting observation that functional bindings sites for both NF-κB and AP-1 are required for full hBD-2 promoter activation upon stimulation with IL-1β as well with PA. This indicates that endogenous stimuli such as IL-1β as well as exogenous stimuli like bacterial products can activate the same signal transduction pathways. It has been shown that Toll-like receptors respond to different microbial products, and the signaling pathways activated (in particular NF-κB, p38, and JNK kinase pathways) are very similar to that generated by activation of the IL-1 receptor (O'Neill, 2002). Furthermore, it has been demonstrated that keratinocytes express Toll-like receptors (Baker *et al.*, 2003; Mempel *et al.*, 2003; Pivarcsi *et al.*, 2003), and Nagy *et al.* (2005) have shown that *Propionibacterium acnes*-mediated hBD-2 induction in keratinocytes is Toll-like receptor-2 and Toll-like receptor-4 dependent. Therefore, one could speculate that induction of hBD-2 through PA may be meditated by the activation of Toll-like receptors. However, this hypothesis remains to be proven.

It is likely that other transcription factors may participate in the hBD-2 induction by IL-1β and PA in keratinocytes. However, our data clearly demonstrate that activation and binding of NF-κB and AP-1 to the hBD-2 promoter are essential events in the signal cascade that mediate the upregulation of hBD-2 through its strong inducers PA and IL-1β. Dysregulation of this cascade may lead to insufficient inducible expression of hBD-2 in skin and may increase the risk of cutaneous infections. A better understanding of the pathways leading to induction of antimicrobial proteins may result in therapeutical approaches that enhance the innate epithelial defense system.

## MATERIALS AND METHODS

### Cell culture and stimulation

Foreskin-derived keratinocytes were isolated from neonatal foreskins and cultured in keratinocyte growth medium (Clonetics, San Diego, CA) in 5% $CO_2$ at 37°C as described (Wiedow *et al.*, 1998). The study was conducted according to the Declaration of Helsinki Principles, and the isolation of human keratinocytes from foreskin was approved by the Institutional Review Board of the University of Kiel. For stimulation experiments, cells were seeded in 12-well tissue culture plates (4 $cm^2$/well; BD Biosciences, San Jose, CA) and used at 70–80% confluence. Cells were stimulated with IL-1β (20 ng/ml) or supernatants of PA (20 μl/ml) for 10 hours. The MAP kinase inhibitors PD 98059, SB 202190, and SP600125 as well as the NF-κB inhibitor Helenalin were dissolved in DMSO. These inhibitors were added directly to the culture medium at a final concentration of 20 μM, 1 hour prior to stimulation. The IL-1ra was dissolved in water and added to the culture medium at a final concentration of 300 ng/ml, 1 hour prior to stimulation.

IL-1β and IL-1ra were purchased from Cell Concepts (Freiburg, Germany) and the inhibitors were from Calbiochem (Darmstadt, Germany). Supernatants of PA (ATCC 33354) were generated by centrifugation (6,000 × *g*, 15 minutes) of a 50 ml overnight culture grown at 37°C in trypticase-soy broth. The harvested bacteria were resuspended in 50 ml MG medium (40 mM $K_2HPO_4$, 30 mM glucose, 22 mM $KH_2PO_4$, 7 mM $NH_4SO_4$, and 0.5 mM $MgSO_4$) and incubated in 500 $cm^2$ triple flasks (Nunc, Wiesbaden, Germany) at 37°C for 24 hours. After incubation, bacteria were removed by centrifugation (6,000 × *g*, 15 minutes) and the resulting supernatant was sterile filtered (0.2 μm) and stored until use at −20°C.

### Real-time PCR

After stimulation, total RNA was isolated and reverse transcribed using standard reagents (Gibco-BRL, Karlsruhe, Germany). The resulting cDNA served as a template in a real-time PCR reaction in a fluorescence temperature cycler (LightCycler, Roche Molecular Biochemicals, Mannheim, Germany). Amplification of hBD-2 and of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was performed as described previously (Harder *et al.*, 2004). A standard curve was plotted for each primer set with the critical threshold cycle ($C_t$) values obtained from amplification of 10-fold dilutions of cDNA to calculate the PCR efficiency of the primer set. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal reference to normalize the target transcripts and a mathematical model described by Pfaffl (2001) was used to determine the relative quantification of hBD-2 gene expression compared with the glyceraldehyde-3-phosphate dehydrogenase gene expression.

### hBD-2 reporter plasmid construction

To analyze hBD-2 promoter activity, 2,338 bp of the human hBD-2 promoter was amplified using the primers 5′-CAGTACAGCAG CAGTGATAG-3′ and 5′-GGGGAGGACATCAAGCCTT-3′. The amplification product was subcloned into the promoterless pGL3-basic *firefly* luciferase vector (Promega, Madison, WI) to generate an hBD-2-2,338-luc reporter plasmid.

The functional role of putative binding sites for the transcription factor NF-κB as well as for the transcription factor AP-1 in the hBD-2 promoter region was studied by site-directed mutagenesis. The ''QuickChange™ XL Site-Directed Mutagenesis Kit'' (Stratagene, La Jolla, CA) was used as indicated by the supplier for the introduction of mutations in the hBD-2-2,338-luc plasmid. The first two of the three putative NF-κB binding sites at positions −205 to −186, −596 to −572 as well as the putative AP-1 binding site at positions −127 to −121 were mutated using mutant primers as described (Tsutsumi-Ishii and Nagaoka, 2003; Wehkamp *et al.*, 2004). For mutating the third NF-κB binding site (positions −2,193 to −2,184), the following primers were used: sense 5′-gtcacaccat cttttgttac tttACgCGtt ccccagc tat gttcaataat-3′ and antisense 5′-attattgaac atagctgggg aaCGcGTaaa gtaacaaaag atggtgtgac-3′. The resulting hBD-2-pGL3 plasmids containing mutated NF-κB and AP-1 binding sites were termed NF-κB-mut1-luc (containing one mutated NF-κB binding site at positions −205 to −186), NF-κB-mut2-luc (containing one mutated NF-κB binding site at positions −596 to −572), NF-κB-mut3-luc (containing one mutated NF-κB binding site at positions −2,193 to −2,184), NF-κB-mut1 + 2 + 3-luc (containing three mutated NF-κB binding sites), AP1-mut-luc (containing a mutated AP-1 binding site), and AP-1 + NF-κB-mut-luc (containing three mutated NF-κB binding sites and a mutated AP-1 binding site). An overview showing the used promoter constructs is given in Figure 2a.

### Transfection and determination of promoter activity

For hBD-2 promoter studies, keratinocytes were seeded in 12-well plates (BD Biosciences) and used for transfection at 60–80%

confluence. Cells were transfected with 0.5 μg of the indicated hBD-2 reporter plasmids and 0.025 μg of an internal control *renilla* luciferase expression plasmid (phRG-TK; Promega) using 1 μl transfection reagent Fugene 6 (Roche Diagnostics Gmbh, Mannheim, Germany) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were stimulated with the indicated stimulus for 10 hours in fetal calf serum-free medium. After stimulation, cells were harvested with 500 μl passive lysis buffer (Promega), and *firefly* luciferase activity from hBD-2-pGL3 reporter vector and *renilla* luciferase activity were measured by the Dual Luciferase assay system (Promega) on a TD-20/20 luminometer (Turner Design, Sunnyvale, CA). Promoter activity was reported as the ratio between *firefly* and *renilla* luciferase activities in each sample.

### hBD-2-ELISA

For ELISA, 96-well immunoplates (MaxiSorp™, Nunc, Roskilde, Denmark) were coated at 4°C for 20 hours with 50 μl (1 μg/ml) goat anti-hBD-2 antibody (Cell Concepts, Umkirch, Germany) in 0.05 M carbonate buffer, pH 9.6. Subsequently, wells were blocked with 200 μl 1% BSA in phosphate-buffered saline (PBS) for 10 minutes at room temperature. After washing three times with 200 μl PBS + 0.1% Tween 20, 100 μl/well of cell culture supernatants and serial dilutions of natural skin-derived hBD-2 in cell culture medium were incubated for 30 minutes at room temperature. Plates were washed thrice with PBS + 0.1% Tween 20, and wells were incubated for 30 minutes at room temperature with 50 μl of biotinylated goat anti-hBD-2 antibody (Cell Concepts) diluted 1:2,500 to 0.2 μg/ml in PBS + 0.1% Tween 20. Plates were washed again three times with PBS + 0.1% Tween 20 and filled with 50 μl/well of Streptavidin–POD (Roche Diagnostics; 1:10,000 in PBS + 0.1% Tween 20). The plates were then incubated for 30 minutes at room temperature, washed three times as described above, and incubated with 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (Roche Diagnostics) as the development agent for 15 minutes at room temperature in the dark. Absorbance was measured at 405 nm with a multichannel photometer (Sunrise; Tecan, Crailsheim, Germany).

### Transcription factor ELISA

Primary keratinocytes stimulated with PA culture supernatant or IL-1β were harvested and nuclear extract was prepared using standard reagents (NucBuster, Novagen, Germany). For transcription factor ELISAs, reagents of two commercially available transcription factor ELISA kits (TransAM, Active Motif, Rixensart, Belgium and NoShift, Novagen/Merck Biosciences, Bad Soden, Germany) were used. The 96-well streptavidin plates (Merck Biosciences) were coated with double-stranded 3′-biotinylated oligonucleotides, matching the sequences of one of the three putative NF-κB binding sites in the hBD-2 promoter (NF-κB-I: 5′-gaggaagggattttctggggtttcctgagtc-3′; NF-κB-II: 5′-gaagatggggagtttcaggggaactttcacataaa-3′; NF-κB-III: 5′-actttg ggacttccccagct-3′). This binding step was performed, using the preparation and binding solution supplied with the NoShift-Kit (Novagen), according to the manufacturer's instructions. Subsequently, reagents of the TransAM-NF-κB-Kit (Active Motif) were used according to the manufacturer's protocol using a self-coated streptavidin plate. Briefly, the bound oligonucleotides were first incubated with 5 μg of the indicated nuclear extract and treated with a primary rabbit antibody against the NF-κB subunits p50, p52, p65, B-Rel, or RelC (provided with TransAM-NF-κB-Kit, Active Motif). After incubation with a secondary horseradish peroxidase-labeled anti-rabbit antibody (Active Motif) and development with the supplied developing reagent, absorbance was measured at a wavelength of 450 nm. This was referenced to 610 nm in a multichannel photometer (Sunrise; Tecan, Crailsheim, Germany).

## CONFLICT OF INTEREST

The author states no conflict of interest.

## ACKNOWLEDGMENTS

We thank K. Schultz for excellent technical assistance and Paul A.J. Russo (The Royal North Shore Hospital, Sydney, Australia), Hiutung Chu, and Jan Wehkamp (University of California, Davis, CA) for help with the manuscript. This work was supported by Deutsche Forschungsgemeinschaft (SFB 617).

## REFERENCES

Baker BS, Ovigne JM, Powles AV, Corcoran S, Fry L (2003) Normal keratinocytes express Toll-like receptors (TLRs) 1, 2 and 5: modulation of TLR expression in chronic plaque psoriasis. *Br J Dermatol* 148:670–9

Christophers E, Henseler T (1987) Contrasting disease patterns in psoriasis and atopic dermatitis. *Arch Dermatol Res* 279(Suppl):S48–51

Diamond G, Zasloff M, Eck H, Brasseur M, Maloy WL, Bevins CL (1991) Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. *Proc Natl Acad Sci USA* 88:3952–6

Florin L, Hummerich L, Dittrich BT, Kokocinski F, Wrobel G, Gack S *et al.* (2004) Identification of novel AP-1 target genes in fibroblasts regulated during cutaneous wound healing. *Oncogene* 23:7005–17

Glaeser R, Harder J, Lange H, Bartels J, Christophers E, Schroeder JM (2005) Antimicrobial psoriasin (S100A7) protects human skin from *Escherichia coli* infection. *Nat Immunol* 6:57–64

Grone A (2002) Keratinocytes and cytokines. *Vet Immunol Immunopathol* 88:1–12

Harder J, Bartels J, Christophers E, Schroeder JM (1997) A peptide antibiotic from human skin. *Nature* 387:861

Harder J, Meyer-Hoffert U, Wehkamp K, Schwichtenberg L, Schroeder JM (2004) Differential gene induction of human beta-defensins (hBD-1, -2, -3, and -4) in keratinocytes is inhibited by retinoic acid. *J Invest Dermatol* 123:522–9

Harder J, Schroeder JM (2005) Psoriatic scales: a promising source for the isolation of human skin-derived antimicrobial proteins. *J Leukocyte Biol* 77:476–86

Hobbie S, Chen LM, Davis RJ, Galan JE (1997) Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella typhimurium* in cultured intestinal epithelial cells. *J Immunol* 159:5550–9

Huang GT, Zhang HB, Kim D, Liu L, Ganz T (2002) A model for antimicrobial gene therapy: demonstration of human beta-defensin 2 antimicrobial activities *in vivo*. *Hum Gene Ther* 13:2017–25

Kelly D, Conway S (2005) Bacterial modulation of mucosal innate immunity. *Mol Immunol* 42:895–901

Krisanaprakornkit S, Kimball JR, Dale BA (2002) Regulation of human beta-defensin-2 in gingival epithelial cells: the involvement of mitogen-activated protein kinase pathways, but not the NF-kappaB transcription factor family. *J Immunol* 168:316–24

Kupper TS, Groves RW (1995) The interleukin-1 axis and cutaneous inflammation. *J Invest Dermatol* 105:62S–6S

Liu AY, Destoumieux D, Wong AV, Park CH, Valore EV, Liu L *et al.* (2002) Human beta-defensin-2 production in keratinocytes is regulated by interleukin-1, bacteria, and the state of differentiation. *J Invest Dermatol* 118:275–81

Liu L, Roberts AA, Ganz T (2003) By IL-1 signaling, monocyte-derived cells dramatically enhance the epidermal antimicrobial response to lipopolysaccharide. *J Immunol* 170:575–80

Liu L, Wang L, Jia HP, Zhao C, Heng HH, Schutte BC *et al.* (1998) Structure and mapping of the human beta-defensin HBD-2 gene and its expression at sites of inflammation. *Gene* 222:237–44

Mempel M, Voelcker V, Kollisch G, Plank C, Rad R, Gerhard M *et al.* (2003) Toll-like receptor expression in human keratinocytes: nuclear factor kappaB controlled gene activation by *Staphylococcus aureus* is toll-like receptor 2 but not toll-like receptor 4 or platelet activating factor receptor dependent. *J Invest Dermatol* 121:1389–96

Nagy I, Pivarcsi A, Koreck A, Szell M, Urban E, Kemeny L (2005) Distinct strains of *Propionibacterium acnes* induce selective human beta-defensin-2 and interleukin-8 expression in human keratinocytes through toll-like receptors. *J Invest Dermatol* 124:931–8

Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA *et al.* (2001) Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 414:454–7

Nomura I, Goleva E, Howell MD, Hamid QA, Ong PY, Hall CF *et al.* (2003) Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J Immunol* 171:3262–9

Ogushi K, Wada A, Niidome T, Mori N, Oishi K, Nagatake T *et al.* (2001) *Salmonella enteritidis* FliC (flagella filament protein) induces human beta-defensin-2 mRNA production by Caco-2 cells. *J Biol Chem* 276:30521–6

O'Neil DA, Porter EM, Elewaut D, Anderson GM, Eckmann L, Ganz T *et al.* (1999) Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. *J Immunol* 163:6718–24

O'Neill LA (2002) Signal transduction pathways activated by the IL-1 receptor/toll-like receptor superfamily. *Curr Top Microbiol Immunol* 270:47–61

Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T *et al.* (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med* 347:1151–60

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45

Pivarcsi A, Bodai L, Rethi B, Kenderessy-Szabo A, Koreck A, Szell M *et al.* (2003) Expression and function of Toll-like receptors 2 and 4 in human keratinocytes. *Int Immunol* 15:721–30

Schroeder JM, Harder J (1999) Human beta-defensin-2. *Int J Biochem Cell Biol* 31:645–51

Sorensen OE, Cowland JB, Theilgaard-Monch K, Liu L, Ganz T, Borregaard N (2003) Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. *J Immunol* 170:5583–9

Sorensen OE, Thapa DR, Rosenthal A, Liu L, Roberts AA, Ganz T (2005) Differential regulation of {beta}-defensin expression in human skin by microbial stimuli. *J Immunol* 174:4870–9

Tsutsumi-Ishii Y, Nagaoka I (2002) NF-kappa B-mediated transcriptional regulation of human beta-defensin-2 gene following lipopolysaccharide stimulation. *J Leukocyte Biol* 71:154–62

Tsutsumi-Ishii Y, Nagaoka I (2003) Modulation of human beta-defensin-2 transcription in pulmonary epithelial cells by lipopolysaccharide-stimulated mononuclear phagocytes via proinflammatory cytokine production. *J Immunol* 170:4226–36

Vitiello M, D'Isanto M, Galdiero M, Raieta K, Tortora A, Rotondo P *et al.* (2004) Interleukin-8 production by THP-1 cells stimulated by *Salmonella enterica* serovar *Typhimurium porins* is mediated by AP-1, NF-kappaB and MAPK pathways. *Cytokine* 27:15–24

Wada A, Ogushi K, Kimura T, Hojo H, Mori N, Suzuki S *et al.* (2001) *Helicobacter pylori*-mediated transcriptional regulation of the human beta-defensin 2 gene requires NF-kappaB. *Cell Microbiol* 3:115–23

Wehkamp J, Harder J, Wehkamp K, Wehkamp-von Meissner B, Schlee M, Enders C *et al.* (2004) NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: a novel effect of a probiotic bacterium. *Infect Immun* 72:5750–8

Whitmarsh AJ, Davis RJ (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med* 74:589–607

Wiedow O, Harder J, Bartels J, Streit V, Christophers E (1998) Antileukoprotease in human skin: an antibiotic peptide constitutively produced by keratinocytes. *Biochem Biophys Res Commun* 248:904–9